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14. ABSTRACT

Our first granting quarter was spent getting set-up administratively as well as logistically and starting our rabbit ear vein studies. Our second and third granting quarters were spent completing the rabbit ear vein studies and starting our pig model experiments. Previous studies had showed a local irritation at the site of injection and it was thought to be associated with the hyper-tonicity of the study drug 4M beta-hydroxybutyrate, 43mM melatonin in 20% DMSO (BHB/M), but no follow-up study had been performed. Our main finding from the rabbit ear vein studies was that BHB/M could be given I.V. at the concentrations stated above if the BHB/M was administered at pH 7.4. Our pig experiments ensued soon after the confirmation from the pathologist regarding our rabbit ear vein findings. After the completion of 18 experiments, it was noted that the study animals did not respond to drug administered intra-osseous in the same manner as animals receiving the drug intra-venous. HPLC analysis of the BHB and melatonin concentrations showed that animals receiving BHB/M intra-osseous did not have the same serum concentrations of BHB and melatonin when compared to animals receiving BHB/M intra-venous.

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Introduction:

Blast injuries have been responsible for the majority of combat deaths in Iraq and Afghanistan, and the likelihood of being exposed to explosives is increasing for military personnel and civilians alike in war zones and other regions of political conflict. The injuries sustained are often accompanied by severe blood loss, and shock from this blood loss is the most common cause of potentially salvageable deaths from combat related injuries.

D-beta hydroxybutyrate and melatonin (BHB/M) is a novel therapy designed to prolong survival in patients who are risk for bleeding to death. The overall strategy is to use strategies learned from study of hibernating mammals to survive a potentially life-threatening blood loss and allow survival to reach effective medical care. BHB/ M includes both an alternate fuel source for cells (D-beta hydroxybutyrate) and a powerful anti-oxidant, melatonin, to protect cells against damage.

Our goal is to evaluate BHB/M in animal models of injury that simulate the battlefield casualty. Our previous work has shown increased survival for animals treated with BHB/M in both rats and pigs. We wish to prove that BHB/M is a safe and effective therapy that can decrease mortality and improve outcomes for injured casualties suffering from polytrauma and blast injuries.

Body:

Task 1: Assess 3 dosing levels and two control groups to address the dose response relationship for the toxicity of the drug product via I.V. infusion in a rabbit ear vein (6 groups; 2 ears/group , n=24 ears. Two sacrifice time points (24 hours and 72 hours), n=12 ears at 24 hours and 12 ears at 72 hours.

Previous studies had shown a local irritation of the vein and tissue at the site of injection of BHB/M (1). The aim of our rabbit study was to establish the non-observable adverse event level (NOAEL) of BHB/M at the injection site when administered intravenously. We wished to assess four dosing levels along with two control groups to address the dose response relationship for the toxicity of the drug product utilizing intravenous (IV) infusion in a rabbit ear vein as well as establish NOAEL (Table 1).

Table 1. Experimental Design for Task 1

Randomization	Euthanasia 24 hours	Euthanasia 72 hours
Normal Saline	2	2
20% DMSO	2	2
BHB/M 1:10	2	2
BHB/M 1:5	2	2
BHB/M 1:2	2	2
BHB/M 1:1	2	2

Rabbit ear veins were infused with normal saline, 20% DMSO, and 2M test solution or 4M test solution (both test solutions with DMSO vehicle). At 24 or 48 hours post administration the rabbit ears were fixed in 10% formalin.

Findings:

We identified NOAEL with the following groups of animals (Table 2). No grossly identified adverse events or venous thrombosis were noted in any of the groups. Tissues were fixed in 10% formalin and processed using H&E staining. Samples were evaluated with histology, read by a blinded, licensed veterinary pathologist utilizing the grading scale in Table 3.

Table 2. Completed Randomization Groups

Randomization	Euthanasia 24 hours	Euthanasia 72 hours
Normal Saline	2	2
20% DMSO	2	2
BHB/M 1:2	2	2
BHB/M 4M	2	2

Table 3. Histological Grading Scale

Grade	Scale
1	Minimal
2	Mild
3	Moderate
4	Marked

Summary of histopathology findings compared by group

Saline versus DMSO injections

- Saline injections, in general, were associated with relatively benign lesions, e.g., perivascular hemorrhage
- DMSO may cause more significant lesions (vascular damage and thrombosis, dermal leukocyte infiltrates etc.), presumably if it leaks extravascularly
- Necrosis and inflammation involving the ear tip is considered to be a more severe manifestation of vascular damage associated with the injection
- Lesions with DMSO appear to be somewhat less severe at 72 hours

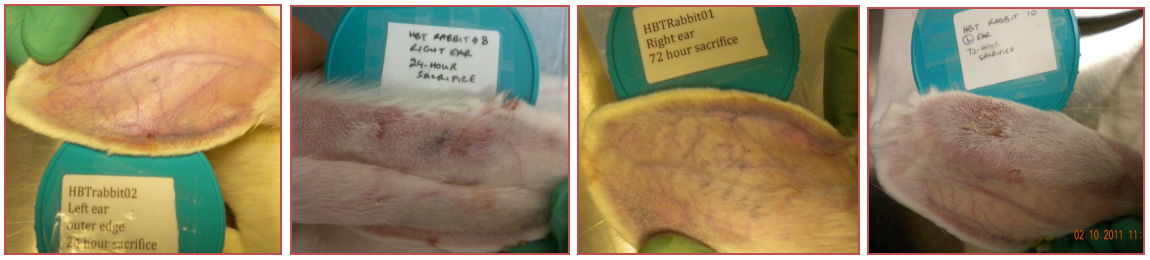
2M test solutions versus DMSO injections

- Although DMSO induced similar lesions to the 2M test solution, it appears that 2M test solution is more likely to cause vascular necrosis and inflammation (noted at 24 hours).
- Necrosis and inflammation involving the ear tip was observed at a higher incidence with the 2M solution.

4M test solutions versus DMSO injections

- Although DMSO induced similar lesions to the 4M test solution, it appears that 4M test solution is more likely to cause vascular necrosis and inflammation (noted at 24 and 72 hours)(Figure 1).

Figure 1. Gross appearance of rabbit ears with no obvious necrosis at 24h or 72h



Comment

Microscopic findings were observed with all solutions injected. These were considered to be more severe (e.g., vascular damage and dermal inflammation) with DMSO than the benign lesions (e.g., perivascular hemorrhage) observed with normal saline, but were not present in all samples suggesting that more severe lesions may be associated with vascular leakage of the DMSO. There appears to be a tendency for slightly greater vascular damage with the 2M and 4M test solutions than with DMSO, however a study with larger numbers would be needed to confirm that this is indeed the case. The presence of necrosis of the ear tips in a number of animals treated with DMSO vehicle, 2M test solution or 4M test solution is considered to be a reflection of more severe vascular damage (e.g., vascular necrosis and thrombosis in the affected animals. In general, lesions appear to have ameliorated at 72 as opposed to 24 hours post administration.

With the pathology supporting our decision to end further rabbit ear vein studies, we proceeded with our pig studies using 4M BHB/M (pH 7.4) for our infusion treatment.

Conclusion:

A pH-neutral mixture of 4M-d-beta Hydroxybutyrate and 43mM-Melatonin solution is not associated with long-term severe vascular or tissue necrosis and is safe to administer via peripheral vein.

Task 3: Assessment of BHB/M administered either intraosseously (IO) or intravenously (IV). 3 study groups: 1 dose based on Task 1, 6 animals/group (3 males and 3 females) and, n=36. Two sacrifice time points (day 2 and day 14), n=18 animals sacrificed on day 2 and n=18 animals sacrificed on day 14.

Task 3 utilized our pig model of tissue injury and hemorrhagic shock utilizing animals randomized based on the following experimental grid (Table 4).

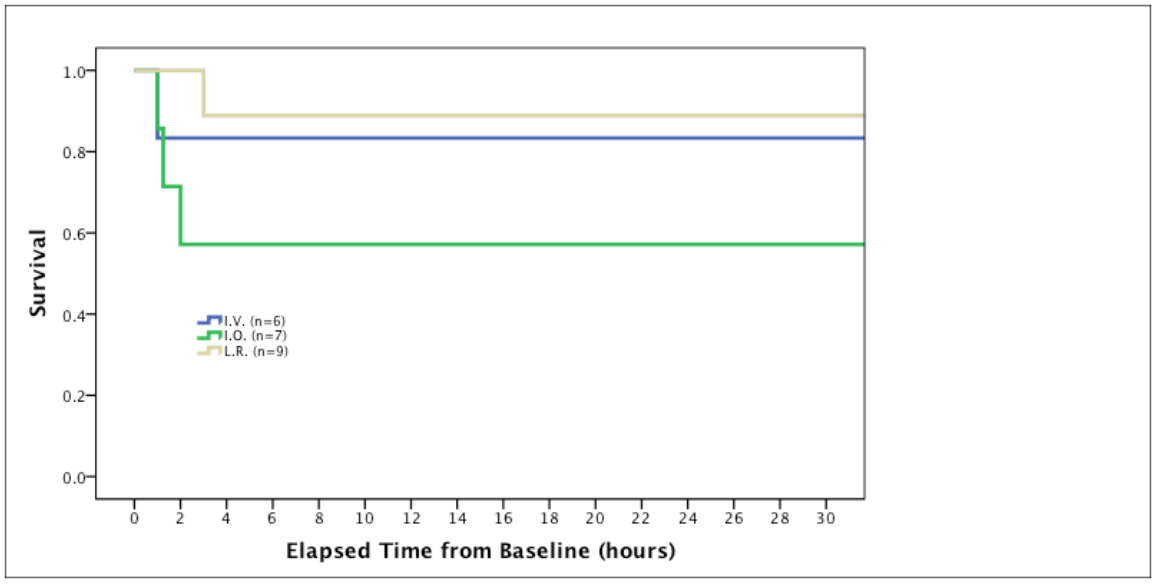
Table 4. Experimental outline of the proposed pig experiments.

Treatment	Number of Animals Sacrificed	
	2 days	14 days
Lactated Ringers'	3 male, 3 female	3 male, 3 female
I.V. BHB/M @ 4M	3 male, 3 female	3 male, 3 female
I.O. BHB/M	3 male, 3 female	3 male, 3 female

To date (10/06/11) we have completed 24 experiments and are on target to complete the remaining 12 experiments by December 2011.

Findings:

Figure 2. Kaplan-Meier survival curve.



Survival analysis noted a difference, currently not significant (p=0.61), between animals receiving BHB/M I.V. and I.O. (Figure 2). Analysis of the BHB and Melatonin concentrations

via HPLC showed that there was ~40% less BHB (Figure 3A) and melatonin (Figure 3B) in serum samples from animals receiving the drug I.O. versus I.V. administration. Analysis of BHB and melatonin concentrations on the additional samples procured is ongoing.

Figure 3A. BHB concentrations during shock and resuscitation (Timepoint key is Table 5).

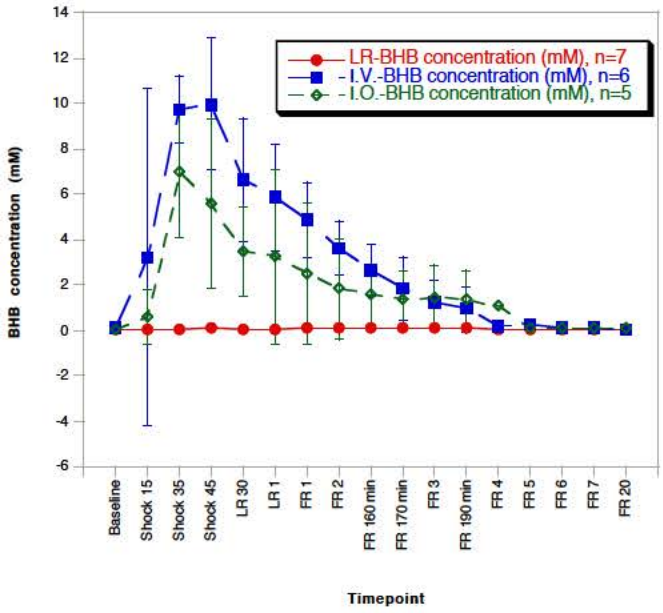


Figure 3B. Melatonin concentrations during shock and resuscitation (Timepoint key is Table 5).

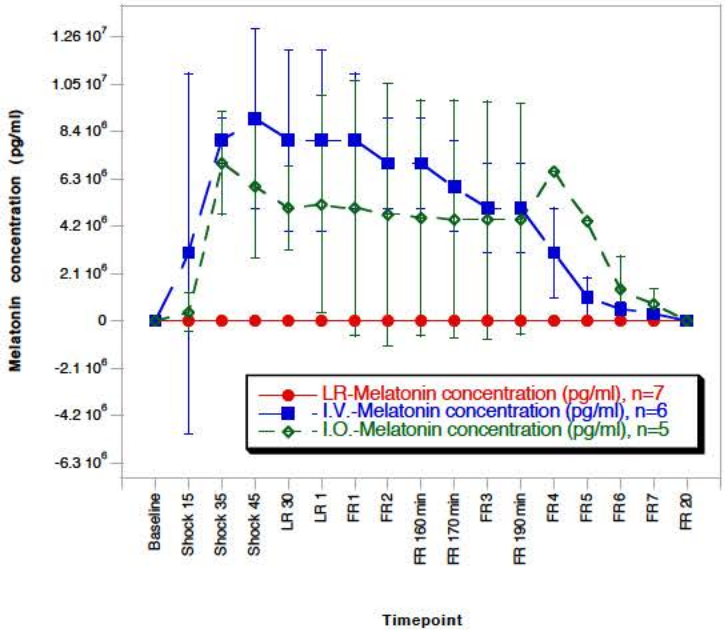


Table 5: Timepoints defined, Limited Resuscitation (LR)=maintenance of SBP above 80 mmHg, Full Resuscitation (FR)=maintenance of SBP above 90 mmHg, Hgb above 6 and Urine output > 1 cc/kg/hr.

Timepoint	Elapsed time from Baseline
Baseline	0
Shock 15	15 minutes
Shock 35	35 minutes
Shock 45	45 minutes
LR 30	30 minutes from the start of Limited Resuscitation phase, ~1.5 hours from baseline
LR 1	60 minutes from the start of Limited Resuscitation phase, ~2 hours from baseline
FR 1	1 hour from the start of Full Resuscitation, 2 hours from the start of Limited Resuscitation, ~3 hours from Baseline
FR 2	2 hour from the start of Full Resuscitation, 3 hours from the start of Limited Resuscitation, ~4 hours from Baseline
FR 160	160 minutes from the start of Full Resuscitation, 3 hours 40 minutes from the start of Limited Resuscitation, ~4.7 hours from Baseline
FR 170	170 minutes from the start of Full Resuscitation, 3 hours 50 minutes from the start of Limited Resuscitation, ~4.83 hours from Baseline
FR 3	3 hour from the start of Full Resuscitation, 4 hours from the start of Limited Resuscitation, ~5 hours from Baseline
FR 190	190 minutes from the start of Full Resuscitation, 4 hours 10 minutes from the start of Limited Resuscitation, ~5.2 hours from Baseline
FR 4	4 hour from the start of Full Resuscitation, 5 hours from the start of Limited Resuscitation, ~6 hours from Baseline
FR 5	5 hour from the start of Full Resuscitation, 6 hours from the start of Limited Resuscitation, ~7 hours from Baseline
FR 6	6 hour from the start of Full Resuscitation, 7 hours from the start of Limited Resuscitation, ~8 hours from Baseline
FR 7	7 hour from the start of Full Resuscitation, 8 hours from the start of Limited Resuscitation, ~9 hours from Baseline
FR 20	20 hour from the start of Full Resuscitation, 21 hours from the start of Limited Resuscitation, ~22 hours from Baseline

Key Research Accomplishments:

- Completed rabbit ear vein studies. 4M BHB/M can be administered I.V. if given at pH 7.4 .
- 50% completion of Task 3 studies. Our data to date supports that BHB/M given I.V. is in circulation at higher concentrations from the onset of bolus compared to BHB/M given I.O and confirms that BHB/M administration improves survival in this model.

Reportable Outcomes:

- Rabbit ear vein studies were presented at ATACCC in the Advanced Technology Applications for Combat Casualty Care in August 15-18, 2011 in Fort Lauderdale, FL.

Conclusion:

-A pH-neutral mixture of 4M-d-beta Hydroxybutyrate and 43mM-Melatonin solution is not associated with long-term severe vascular or tissue necrosis and is safe to administer via peripheral vein.

-Based on the measurements of BHB and melatonin via HPLC. We will be proposing (Figure 4) to add a study arm to the current protocol that would allow us to infuse different doses of BHB/M intraosseously to give us futher insight into this matter.

Figure 4: Table outlining proposed study groups for assessment of BHB and Melatonin given at different doses either I.V. or I.O.

Group	Drug Component	Concentration of Drug component	Number of animals requested
1	Lacated Ringers'	10cc/kg, 0.66 cc/kg/hr	4
2	BHB/M ½ dose I.V.	4 M BHB/ 43 mM melatonin	4
3	BHB/M Full dose I.V.	4 M BHB/ 43 mM melatonin	4
4	BHB/M Double dose I.V.	4 M BHB/ 43 mM melatonin	4
5	BHB/M ½ dose I.O.	4 M BHB/ 43 mM melatonin	4
6	BHB/M Full dose I.O.	4 M BHB/ 43 mM melatonin	4
7	BHB/M Double dose I.O.	4 M BHB/ 43 mM melatonin	4

4 additional animals are requested for unforeseen experimental complications.

I.V.= intravenous

I.O.=intraosseous

References:

1. Mulier KE, Lexcen DR, Luzcek E, Greenberg JJ, Beilman GJ. Treatment with beta-hydroxybutyrate and melatonin is associated with improved survival in a porcine model of hemorrhagic shock. Resuscitation (2011), in press.